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XXX CICLO

Integration of cAMP signaling and the ubiquitin system in the control of primary cilium

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ABSTRACT

The primary cilium is an antenna-like sensory organelle able to receive extracellular signals and it is localized on the surface of most human cells.

In my thesis, I investigated the connection between G-protein coupled receptor (GPCR) signaling and the ubiquitin proteasome system (UPS) pathway in the control of cilium stability. I identified, at pericentriolar region, a trimeric complex composed by PCM1, NEK10 and PKA. I demonstrated that NEK10 has a crucial role for ciliogenesis. Phosphorylation by PKA primes NEK10 to proteasomal degradation. Disappearance of NEK10 promotes cilia resorption. I identified CHIP as the E3 ubiquitin ligase responsible of NEK10 ubiquitination and I demonstrated that CHIP mediates the effects of cAMP on primary cilium stability.

Dearangement of this control mechanism was observed in proliferative and genetic disorders. Collectively, the findings unveil a pericentriolar kinase signalosome that efficiently links the cAMP cascade with the ubiquitin-proteasome system, controlling essential aspects of ciliogenesis.

1 INTRODUCTION

1.1 The cAMP-dependent signal transduction pathway

The biological organisms are able to modify a variety of cellular processes to adapt themselves to multiple conditions. Subsequently to a change, the organism communicates to specific target cells through the extracellular messengers such as hormones, neurotransmitters and growth factors.

The binding between a ligand and its specific receptor on the cell surface can lead to a different biophysical response through activation of specific second messengers.

The second messengers trigger a biological response that may consist in the regulation of different cellular processes, as gene transcription, protein translation, hormone production, cellular differentiation and cellular division.

The cyclic AMP (cAMP) is the most famous second messenger because it is involved in a wide array of biological processes[1, 2].

When an extracellular ligand binds a G-protein coupled receptors (GPCR), it starts the cAMP signaling cascade.

The G protein-coupled family receptors (GPCRs) are a large family of trans-membrane proteins that transduce extracellular signals into the cell[3]. The binding of extracellular ligand to its GPCR activates the adenylyl cyclase (AC), an enzyme that converts the ATP in cAMP[4]. The activity of ACs is stimulated by the interaction with the stimulatory α subunit of the G-protein (Gαs). In basal conditions, Gαs forms an heterotrimeric complex with β and γ subunits. Subsequently to the binding of the extracellular messenger, the GPCRs causes the dissociation of heterotrimeric G-proteins, with consequent activation of ACs by the Gαs subunit[5].

In addition to AC, the levels of cAMP are regulated by the cyclic nucleotide phosphodiesterases (PDEs) and phosphatases (PPs).

Phosphodiesterases (PDEs) are involved in the regulation of the intracellular level of cAMP. They are part of a large superfamily of enzymes that hydrolize the 3′ - 5′ phosphodiester bond in the second messenger cAMP with the formation of 5′-AMP[6].

By reducing the levels of cAMP, PDEs regulate the duration and amplitude of the cyclic nucleotide signaling[7]. The subcellular localization of the enzymes is controlled by the N-terminal regulatory region[8]. The distribution of PDEs in the cells generates intracellular micro domains that locally enhance the sensitivity and specificity of the intracellular response to the cAMP[9].

There are three distinct classes of direct effectors of the cAMP : cAMP-dependent protein kinase (PKA), RAP exchange proteins (EPACs), and cAMP gated ion channels (cNGC) (**Fig.1**). Among these effectors, the more studied is Protein kinase A (PKA).

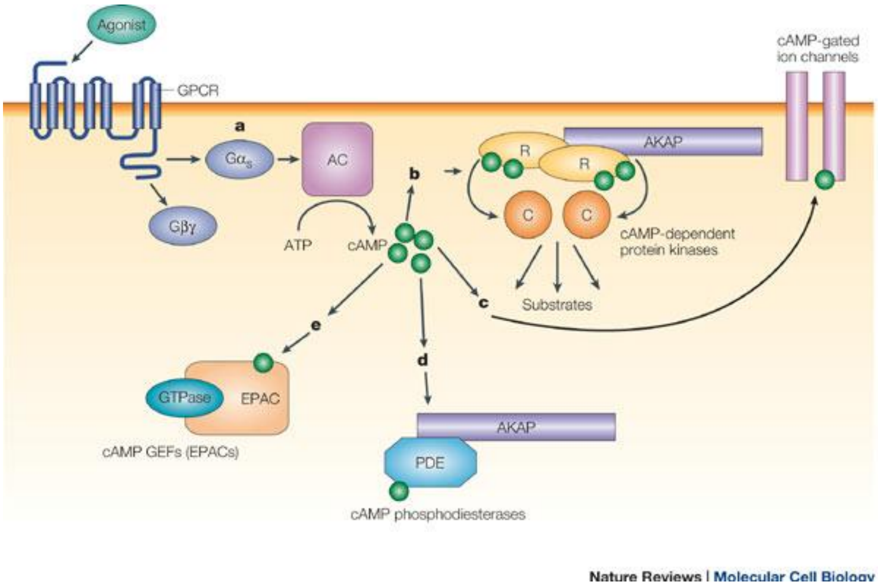


Figure 1. Schematic diagram of cAMP synthesis and downstream effectors activation. When an extracellular ligand binds to and activates a seven-transmembrane G-protein-coupled receptor, the signal is passed through the heterotrimeric G protein to adenylyl cyclase. The activated adenylyl cyclase converts ATP into the second messenger cAMP. Principal effectors of cAMP are PKA, PDE and EPAC. The cyclic nucleotide phosphodiesterases (PDE) degrades the phosphodiester bond in the second messenger molecules cAMP and cGMP. RAP exchange proteins (EPACs) are cAMP-dependent guanine-nucleotide-exchange factors for the small GTPases, and are known to be important mediators of cAMP signaling. Finally there are cAMP gated ion channels (cNGC) that function in response to the binding of cyclic nucleotides as cGMP and cAMP.

1.2 Protein Kinase A (PKA)

Protein kinase A (PKA) is a serine/threonine kinase and it is one of the major effector of the cAMP. Indeed, every time an extracellular stimulus leads to an intracellular response cAMP-mediated, PKA is activated[10].

PKA is a tetrameric protein composed by two catalytic (C) and two regulatory(R) subunits. The whole complex makes the holoenzyme inactive.

In response to the binding of a ligand to GPCRs, there is a quick increase of the intracellular concentration of cAMP generated by the ACs proteins[2]. So cAMP binds to R subunits, this binding causes their dissociation from the catalytic subunits (**fig. 2**). In this way, the catalytic subunits are able to phosphorylate many different downstream cellular substrates that include ion channels and a lot of transcription factors[2].

The biochemical and functional features of PKA holoenzyme are largely determined by the structure, properties and relative abundance of the R subunits[11]. The conserved catalytic core in the C-subunit is encoded from three different genes, Cα, Cβ e Cγ[12]. The catalytic subunit is a 350-amino acid protein and the kinase core is localized into 40-300 residues. The smaller N-terminal lobe is composed by β-sheets and is responsible for nucleotide binding[13], whereas the larger C-terminal lobe is composed by α-helics and it is responsible of substrates binding and catalysis[11].

R-subunits are encoded by four different genes (R1α, R1β, R2α e R2β) that confer the different biochemical and biological characteristics to the PKA isoforms[12]. The R-subunit polypeptide contains an NH_2-terminal dimerization domain, an autophosphorylation site (that is the principal contact site for the C subunit) and two cAMP binding sites. Another functional site present on the N-terminus of the R-subunits is the dimerization/docking (D/D) domain that provides a docking site for the A Kinase Anchoring Proteins (AKAPs) [14]. In addition, the R subunits are able to form both homo-and heterodimers generating a large number of combinations, which further contribute to diversity and presumably specificity in the cAMP signal pathway[15].

The PKAs that contain either RI or RII are identified as PKA type I or type II and they have different sensitivities to cAMP. They also differ for localization and expression. PKA type I, in fact, is largely cytoplasmic, whereas PKA type II is confined to subcellular structures and compartments. Furthermore RIα and RIIα are ubiquitously expressed, RIβ has been mainly abundant in neuronal tissues while RIIβ has the highest expression in neuronal, adipose, testes and heart tissues[12, 16]. Studies demonstrated that ablation of the gene encoding the RIβ leads to deficits in hippocampal long-term depression and depotentiation[17, 18] but with a compensatory increase in total PKA activity, suggesting a unique role for RIβ in synaptic plasticity[19]. A targeted disruption of the RIIα gene yields viable mice with no physiological abnormalities, implying that PKAI and/or PKAIIβ compensates for the RIIα defect[20, 21]. The mutant mices with disruption of the mouse RIIβ gene are lean and have elevated metabolic rates caused by increases in both basal PKA activity and the basal rate of lipolysis [22, 23]. RIIβ KO mice also display defects in neuronal gene expression, learning and behavior[24, 25]. The activity of PKA is regulated by specific protein phosphatases. It has been demonstrated that phosphatases belonging to the PP1 and PP2A families are responsible for dephosphorylation of PKA substrates. In turn, PKA can control phosphatase activity by phosphorylation of specific PP1 inhibitors, such as I-1 and DARPP32[26].

PKA signaling is compartmentalized thanks to AKAPs protein. They are a group of several scaffold proteins that anchor the Rs subunits to tissues and different cellular compartments.[15]

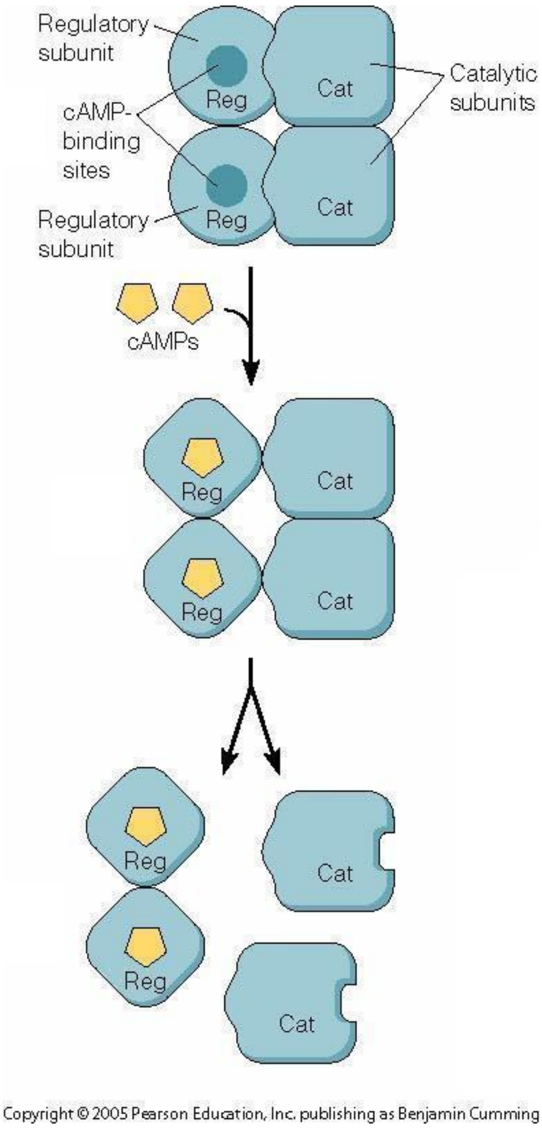


Figure 2. PKA molecular structure and activation mechanism.
When two molecules of cAMP bind the regulatory subunits of PKA, the holoenzyme is disassociates causing the activation of catalytic subunits.

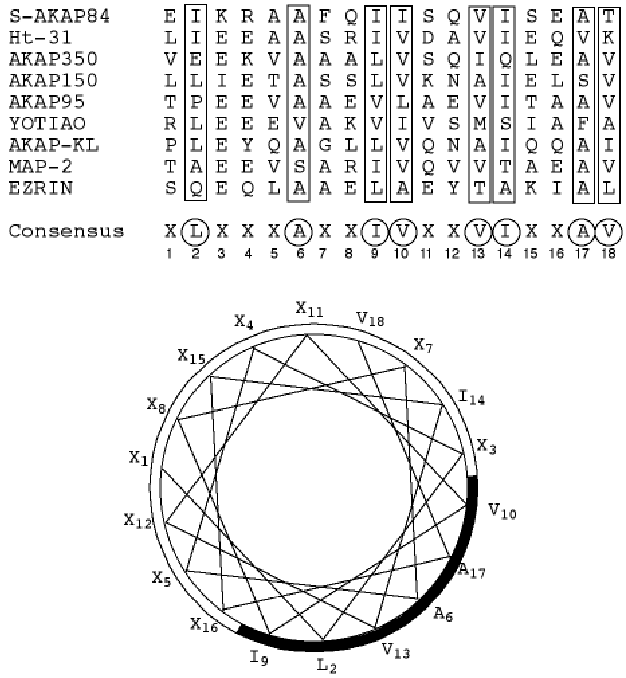
1.3 AKAP proteins

PKA is targeted to specific cellular organelles or subcellular locations through interaction with a family of distinct but functionally homologous proteins called AKAPs (A-Kinase-Anchor-Proteins)[27-29].

AKAPs contain a PKA-binding motif, approximately 14 amminoacids, that are able to bind the R subunit[7, 30]. This region forms an amphipathic helix in which hydrophobic residues are located in the interior face while charged residues align on the exterior surface. This helical wheel binds with high affinity the N-terminal docking/dimerization (D/D) domain of the PKA-R dimer directing it in proximity of its substrates[31] (**Fig. 3**). In the past have been identified several AKAPs that bind both RI and RII subunits[32].

Thought their targeting domain, AKAPs protein have been found in various cellular organelles such as centrosomes, dendrites, endoplasmic reticulum, mitochondria, nuclear membrane, plasma membrane and vesicles. The presence of PKA nearby of its substrates enhances the PKA-dependent phosphorylation of a large number of cellular substrates. Infact the cells that express high levels of PKA are more responsive to signals caused by the intracellular increase of second messenger cAMP[33, 34].

Although AKAPs have been defined on the basis of their interaction with PKA, several of these molecules are able to bind other enzymes such as receptors, effectors, protein phosphatases and kinases. In fact in the cells, AKAPs form macro molecular complexes, named transduceosome, where distinct signaling pathways converge and are attenuated or amplified, improving the specificity and efficiency of biological responses[34-36].



J Mol Biol.The biological functions of A-kinase anchor proteins.

Figure 3. Consensus sequence of AKAP-RII-binding domains.

Consensus sequence derived from the alignment of the primary sequences of several AKAPs and the amphipathic helical wheel and the residues forming it are depicted as a thick line.

1.4 The ubiquitin proteasome system and E3 ubiquitin ligase CHIP

In mammalian cells the post-translational modification of proteins is a common mechanism of cell regulation. The covalent modification of proteins by attachment of other protein is one such example. The control of this cellular mechanism is most important because every injury that affects this mechanism can lead to development of human diseases or disorders, including cancer. The balance between the synthesis and degradation of proteins is regulated by the ubiquitin-proteasome system (UPS).

Ubiquitylation has a central role in several physiological processes and it is involved in the regulation of cell survival, differentiation, genetic integrity, protein quality control and signaling. Frequently, the substrates of ubiquitin are degradated through the proteasome[37].

This process requires the activity of three enzymes: E1 (ubiquiting activating), E2 (ubiquitin conjugating) and E3 (ubiquitin ligating) that act in series to catalyze ubiquitination.

The E1 enzyme is the activating enzyme which ubiquitin is attached to in an ATP-dependent reaction. The E2 enzyme is the conjugating enzyme, which the ubiquitin is transferred to, from the E1. The E3 is the ubiquitin ligase, which directly or indirectly catalyzes the transfer of the ubiquitin to the lysine of a target protein, with the formation of an isopeptide bond[38] (**Figure. 4**).

The ubiquitin substrates are not always directed to degradation via UPS. Infact the amount of ubiquitin tagged protein is balanced through the activity of deubiquitylating enzymes (DUBs) that reverse ubiquitylation by removing conjugated ubiquitin tags[39, 40]. The RING finger domain of E3 ubiquitin ligases contain a characteristic cysteine-rich-zinc-binding domain composed by a pattern of conserved cysteine and histidine residues.

The carboxyl terminus of Hsp70-interacting protein (CHIP), also known as STUB1 (STIP1 homology and U-box containing protein1), is a member of E3 ubiquin ligase that plays an important role in maintenance the protein homeostasis in the cytoplasm[41].

In literature is described that CHIP binds several members of the molecular chaperones Hsp70/90 family that have a central role in the refolding of proteins[42, 43]. Specifically, the principal activity of CHIP is to remove, through the ubiquitin proteasome system, the misfolded or damaged proteins that can lead to development of human cancers or other disorders[44, 45].

The protein CHIP was first characterized in human heart[42]. The important domain that allows the binding between CHIP and Hsp70/90 is a tetratricopeptide repeats domain (TPR) located at N-terminus of the protein, whereas a U-box domain at the C-terminus of CHIP displays the ubiquitin ligase activity.

In literature are described several ciliopathies linked to a loss activity of CHIP, in particular several form of ataxia such as spinocerebellar ataxia autosomal recessive 16 (SCAR16)[46, 47].

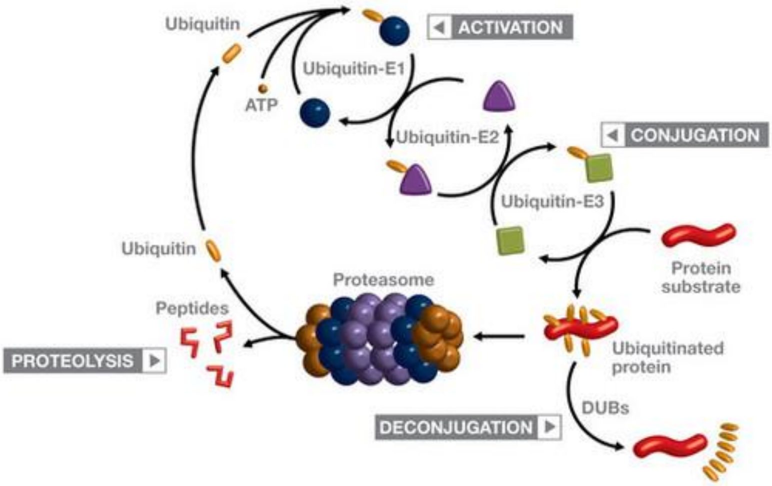


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Figure 4. Schematic representation of ubiquitin system.

The conjugation of ubiquitin molecules to substrates requires coordinated action of three enzymes: the ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme E2 and the E3 ligase that associates the ubiquitin molecules to the substrates. Once ubiquitinated, the proteins can be degraded by the proteasome or de-ubiquitinated by a specific DUBs enzyme.

1.5 Ciliogenesis

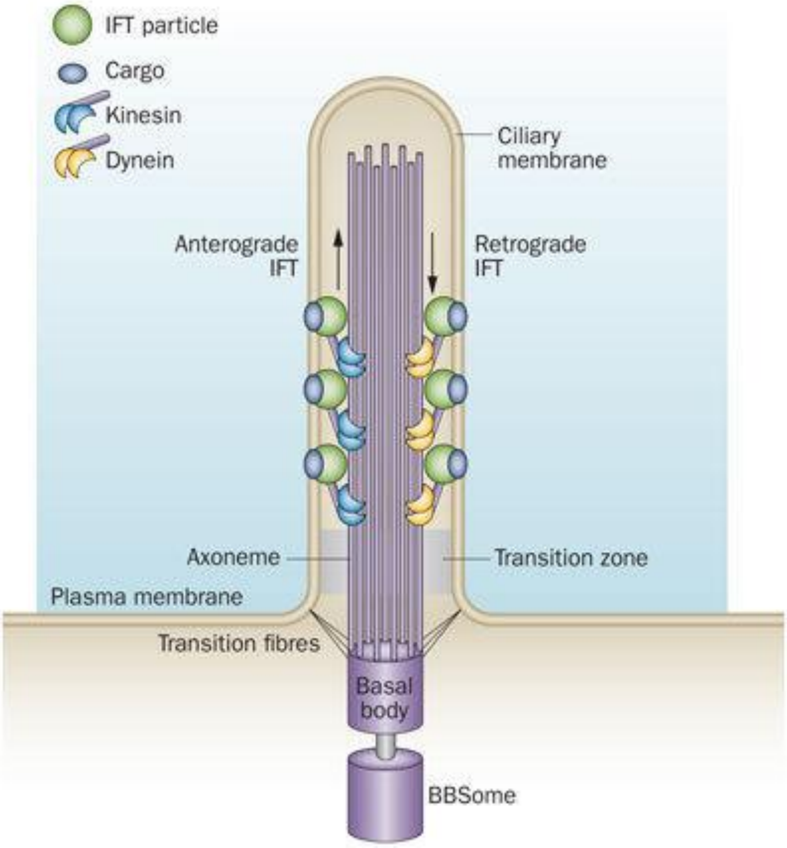
The primary cilium is considered a sensory organelle able to receive extracellular signals and transmit them into cells.

In mammalian cells primary cilia acts as “antennae” to sense signals such as growth factors, hormones, odorants and development morphogens[48, 49]. In the last years the focus on primary cilium has increased since this organelle has a critical role in regulating of different signaling pathways and during vertebrate development and tissue homeostasis[50].

Defects in ciliary assembly or its function can lead to several cilium-related human diseases called ciliopathies[51]. Ciliopathies are a group of genetic diseases caused by alterations of development, functioning and signaling of primary cilium. These syndromes present manifestations as polydactyly, retinal degeneration, mental retardation, anosmia, obesity and kidney cysts. The list of ciliopathies continues to grow and at the present includes Bardet-Biedl Syndrome (BBS), Joubert Syndrome (JS), Oral-facial-digital Type I (OFD1), retinal degeneration, polycystic kidney disease[52].

Primary cilium usually forms during G1 phase of the cell cycle or when cells, deprived of nutrients and mitogens, exit from cell cycle and enter in quiescence state.

The primary cilium grows out from centrosome, the main microtubule-organizing center (MTOC) in animal cells, and it is composed of a microtubule-based core structure called axoneme. The axoneme is nucleated by basal body that includes the mother centriole and associated pericentriolar material (PCM). Moreover the axoneme is surrounded by a ciliary membrane and it is assembled by nine parallel doublet microtubules which elongate from the basal body. The distal region of basal body, where the outer doublets begin to form, is called the transition zone. The ciliary pocket, an invagination of the plasma membrane at the root of the cilium, is found on some types of mammalian cells[53]. **(Fig. 5)**



Nature Neurology.Primary cilia in neurodevelopmental disorders
Figure 5. Structure of primary cilium.

Since the protein synthesis occurs into the cytoplasm, elongation of primary cilium requires the intraflagellar-transport machinery (IFT), two complexes that move themselves within the cilium. IFT complex B is responsible of anterograde transport of ciliary proteins from base to the tip of cilium, whereas IFT complex A is responsible of retrograde transport from tip to base of cilium[53]. In literature is described that IFT complex B is crucial for a correct assembly of primary cilia. In fact the lack of some IFT complex proteins causes short or absent cilia. By contrast, IFT complex A transport back proteins to the basal body but it seems not be crucial for assembly of cilia[54].

In the cells there are a lot of positive and negative regulators that control the correct assembly of primary cilium. Examples of negative regulators are the centriolar protein Cp110 and the kinesin Kif24; they are able to destroy ciliary axoneme, so their abundance is lower during cilia assembly[55, 56]. Conversely, other proteins such as the ser/Thr kinase TTBK2 and MARK4 act to promote cilium formation. Upon serum deprivation, in fact, TTBK2 localizes at basal body where removes Cp110 and recruits IFT complexes[57]. The balance between cilia assembly or disassembly is regulated by also post-translational modifications. HEF1/Cas-L/NEDD9 is a component of focal adhesions that colocalizes with Aurora kinase A at the centrosome. Aurora A stimulates histone deacetylase 6(HDAC6) resulting in deacetylation of axonemal microtubules rendering them unstable[58].

1.6 Correlation between cAMP signaling, the UPS system and primary cilium

In literature is extensively documented the tight correlation between ubiquitylation and cAMP pathway while the linkage that couples the cAMP cascade and primary cilium is a very current topic.

Nevertheless several components of cAMP pathway, including G-protein coupled receptors (GPCRs), adenylate cyclases (ACs) and cAMP-dependent protein kinase A (PKA), conduct different important roles within the ciliary compartment[59].

In literature is documented that a pool of PKA is localized at centrosome, the basal structure of primary cilia[60-62].

In particular, PKA is a negative regulator of hedgehog (Hh) pathway that plays a critical role in embryonic development[63, 64]. In the absence of Hedgehog ligand, PKA phosphorylates Ci/Gli transcription factors promoting their proteolysis and the production of the repressor forms of Ci/Gli blocking Hedgehog target gene expression. In contrast, the activation of Hedgehog signaling increases the active forms of Ci/Gli resulting in Hedgehog target gene expression.

The basal level of PKA activity in Hedgehog-responsive cells is precisely regulated and it is maintained at the basal body of cilium by interacting with A-Kinase-Anchor-Proteins (AKAPs)[62]. Probably, this regulation is conducted by another ciliary G-coupled receptor Gpr161 that, after stimulation of the transmembrane protein Smoothened (Smo), exits from the cilium maintaining inactive the PKA[65].

This strongly suggests the existence of a localized pool of PKA maintained at the base of cilium which targets Hh signaling during the essential steps of ciliogenesis.

cAMP is a second messenger implicate in a wide of biological functions including the activity of several E3 ligases. In this manner, PKA modulating the activity of the principal enzymes of ubiquity proteasome system, it controls the stability, the turnover and the biological activity of several cellular substrates. In neurons, PKA controls the neurite outgrowth, morphogenesis and improve the synaptic plasticity and memory. In response to an increase of intracellular levels of cAMP, the E3 ubiquitin ligase praja2 ubiquitinates and degrades NOGO-A, an important inhibitor of neurite outgrowth in mammalian brain[66].

Another one important correlation between cAMP cascade and UPS system is the regulation of the turnover of regulatory subunits of PKA by E3 ubiquitin ligase praja2. When in the cells there is an increase of cAMP levels, praja2 promote ubiquitylation and subsequent proteolysis via UPS of R subunits, regulate the strength and duration of PKA signal in response to cAMP[67].

This relationship between these two systems suggest the exists of a circuit finely regulated in which cAMP pathway controls the turnover/stability of key elements of metabolic and proliferative pathways, but at the same time UPS regulates the stability of components of the cAMP cascade and the duration and amplitude of its signal[7].

1.7 NimA related kinase 10 (Nek10)

During the years, the scientific research has explained in which way the damages to cell cycle, checkpoint alteration and chromosome instability, can lead to development of cancers and other disorders. NIMA-related kinases (NEK) proteins are serine/threonine kinase, involved in the regulation of cell cycle, were identified in several organisms from protists to multicellular eukaryotes including mice and humans[68, 69]. In literature is described that some members of this family are involved in ciliary functions and ciliopathies[70].

Statistical analysis have confirmed that this family of proteins coevolved with centrioles, which represent the microtubule-organizing center and prime the assembly of basal bodies of cilia[71].

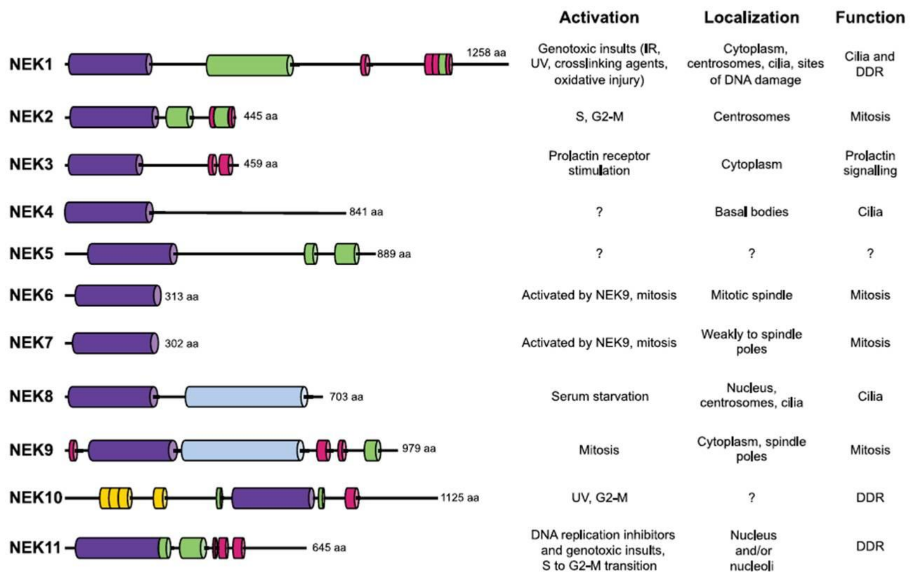
In human cells there are eleven genes that encode from NEK1 to NEK11 proteins. **(Fig. 6)**

Nek10 gene localizes on the short arm of human chromosome 3 (3p24.1). This gene encodes for fourteen transcripts the longest of which has 39 exons. The 4.25 Kbp transcript encodes a protein of 1172 residues with an estimate weight of 133 Kda[72].

Despite low overall sequence homology, the organizational features of NimA are broadly conserved among mammalian Nek kinases. Infact all these proteins are characterized by a N-terminal catalytic domain, except Nek10 that have its kinase domain in the central position. In addition to catalytic domain there are: His-Arg-Asp (HRD) motif which is typical of ser/thr kinases regulated through phosphorylation[73], coiled-coiled domains which mediate the oligomerization and PEST sequences which participate in ubiquitin dependent proteolysis[69].

In contrast with the conserved catalytic domain, the C-terminal region of NEK proteins is different in length, sequence and domain organization. As explained before, several NEK members have important roles in cell cycle control, in particular NEK2 facilities spindle pole separation whereas NEK6, NEK7 and NEK9 are important in generating the mitotic spindle[74, 75]. NEK1 is involved in the repair of DNA strand breaks at G1-S and G2-M transitions[76-78] and NEK10 and NEK11 are involved in G2-M DDR checkpoint.

Specially, NEK10 is required for the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) signaling upon UV irradiation, but not in response to mitogens, such as epidermal growth factor. After the stimulation, NEK10 physically associated with Raf-1 and MEK1 in a Raf-1-dependent manner and the formation of this complex was necessary for Nek10-mediated MEK1 activation. The appropriate maintenance of the G2/M checkpoint following UV irradiation required Nek10 expression and ERK1/2 activation, indicating a role for Nek10 in the cellular response to UV irradiation[68].



Cell Div. Nek family of kinases in cell cycle, checkpoint control and cancer.

Fig.6 The human NIMA-related protein kinase (NEK) family

A schematic representation of human NEKs gene. Are indicated: the kinase domains (purple), coiled-coils (green), degradation motifs (red), RCC1 (regulator of chromatin condensation 1) domains (light blue) and armadillo repeats (yellow)

2 AIM OF THESIS

The cAMP signaling pathway has been carefully studied in the laboratory where I practiced my PhD program. In the last years, our attention was focused on the relationship between ciliogenesis and cAMP pathway, in particular on the regulation of the primary cilium stability, via UPS, in response to GPCR signaling. By a proteomic analysis, we identified PKA as a component of a macromolecular complex that includes the pericentriolar matrix protein 1 (PCM1) and Nima-related Kinase 10 (NEK10). PCM1 is a scaffold protein mostly localized in centriolar satellites and its role about the ciliogenesis is abundantly described[79], whereas the involvement of NEK10 kinase is widely unknown.

During my PhD program, I studied the role of NEK10 in primary ciliogenesis. I found that NEK10 plays a major role in the formation of primary cilium. Activation of GPCR-cAMP signaling causes the disassembly of primay cilium and that this stimulus primes the degradation of NEK10 protein by the ubiquitin proteasome system through E3 ubiquitin ligase CHIP. Disappearance of NEK10 levels leads to cilia resorption, underlying the central role of cAMP-NEK10 axis in the control of primary cilium stability.

Accordingly, the principal aims of my PhD thesis were the following:

1. Identify NEK10 and PKA as novel components of the multimeric signaling complex assembled at pericentriolar region by PCM1.
2. Determine the role and the mechanism of NEK10 and PKA in the control of ciliogenesis.
3. Analyze the intersection between cAMP signaling and NEK10-regulated ciliogenesis.

4. Study the role of NEK10 in the ciliopathies, such as the autosomal recessive spinocerebellar ataxia-16 (SCAR16).

3 MATERIALS AND METHODS

3.1 Cell lines. Human embryonic kidney cell line (HEK293) and primary skin fibroblasts from SCAR16 patients were cultured in DMEM containing 10% fetal bovine serum (FBS) supplemented with 2mM L-glutamine, 100 IU/ml penicillin, in an atmosphere of 5% CO_2 at 37°C.

3.2 Plasmids, siRNAs and transfection. Vectors encoding for NEK10-flag (wild type and mutants) and PCM1-HA were provided by Dr Stambolic V. and Dr Kamiya A. respectively. HA-Ub, CHIP-myc (wild type and K30A mutant), HSP70-V5, were provided by Dr Carlomagno F. and epitope Myc tagged RIIβ vectors were kindley provided by Dr Ginsberg SH. NEK10 phosphorylation mutants (T223A and T812A) were generated by PCR using specific oligonucleotides. siRNAs targeting distinct segments of coding regions of NEK10 and CHIP were purchased from IDT and Life technologies.

The siRNA sequence (IDT) targeting the 3'-UTR (untranslated region) of human NEK10: sense sequence: CCACAAGACAUUAGUAAA UUUACTT antisense sequence: CGGGUGUUCUGUAAUC AUUUAAAUGAA or human CHIP sense sequence: UUACACCAACCGGGCCUUtt; antisense sequence: CAAGGCCC GGUUGGUGUAAta.

siRNAs were transiently transfected using Lipofectamine 2000 (Invitrogen) at a final concentration of 100 pmol/ml of culture medium.

3.3 Antibodies and chemicals. Polyclonal antibodies directed against PCM1 were purchased from ABCAM and Cell Signaling and used at working 1:1000; rabbit polyclonal antibodies directed against phosphoPKA was purchased from Cell Signaling and used at working dilution of 1:1000;

monoclonal antibodies directed against RIIβ was purchased from BD Transduction and used at working 1:2000; haemagglutinin epitope (HA) was purchased from Covance and used at working diluition of 1:1000; monoclonal antibodies directed against flag and myc epitope used at working diluition 1:3000 were purchased from Sigma; polyclonal antibodies directed against acetylated alpha tubulin was purchased from ABCAM. Forskolin was purchased from Sigma.

3.4 Immunoprecipitation and pull down assay. Cells were washed twice with phosphate-buffered saline and lysed in a buffer (50mM TRIS–hydrogen chloride, pH 7.4, 150mM sodium chloride, 5mM magnesium chloride, 5mM dithiothreitol, 1mM ethylene diamine tetraacetic acid, 1% Triton X-100,containing aprotinin (5 μg/ml), leupeptin (10 μg/ml), pepstatin (2 μg/ml), Na3VO4 and 1mM phenylmethylsulfonyl fluoride and protease inhibitors. The lysates were cleared by centrifugation at 15,000 g for 15 min. Cell lysates (2 mg) were immunoprecipitated in rotation at 4 °C overnight with the indicated antibodies. Pellets were washed four times in lysis buffer and eluted in Laemly buffer.An aliquot of whole cell lysates (WCE) (100 μg) or immunoprecipitates were resolved on sodium dodecyl sulfate polyacrylamide gel and transferred on nitrocellulose membrane (Biorad, Milan, Italy) for 3 h. Filters were blocked for 1 h at room temperature in Tween-20 Phosphate buffer saline (TPBS) (PBS- Sigma, 0, 1% Tween 20, pH 7.4) containing 5% non-fat dry milk. Blots were then incubated O/N with primary antibody. Blots were washed three times with TPBS buffer and then incubated for 1 h with secondary antibody (peroxidase-coupled anti rabbit (GE-Healthcare) in TPBS. Reactive signals were revealed by enhanced ECL Western Blotting analysis system (Roche).

GST-fusions were expressed and purified from BL21 (DE3) pLysS cells. GST hybrid proteins immobilized on glutathione beads were incubated for 3 hr with cell lysates from HEK293 cells transiently expressing flag-NEK10 constructs in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 5mM $MgCl_2$, 5mM DDT, 1 mM EDTA, 1% triton X-100) in rotation at 4 °C for 4 hours. Pellets were washed four times in lysis buffer supplemented with NaCl (1 M final concentration) and eluted in Laemmli buffer. Eluted samples were size-fractionated on SDS-PAGE and immunoblotted.

3.5 PKA phosphorylation assay. Cells transfected with either wild type NEK10-flag or with NEK10-flag mutants (T223A-Flag and T812A-Flag) were left untreated or stimulated with FSK (15min). NEK10 was immunopurified with anti-flag antibodies. The precipitates were immunoblotted with anti-flag and with anti-phospho-(K/R)(K/R)X(S*/T*) specific antibodies. The quantification is shown from n=4 independent experiments (± SEM).

3.6 Immunofluorescence and confocal analysis. For immunofluorescence studies, HEK293 cells transiently transfected with the expression vectors were plated on poly-L-lysine (10μg/ml) coated glass coverslips. Cells were fixed with Paraformaldehyde for 20 minutes. After three washes, cells were immunostained with polyclonal antibodies directed against PCM1 was purchased from ABCAM, with polyclonal directed against NEK10 and used at working diluitions of 1:100; monoclonal antibodies directed against RIIβ was purchased from BD Transduction and used at working diluition of 1:400, monoclonal antibodies directed against Flag epitope used at working dilution of 1:400; polyclonal antibodies directed against acetylated alpha tubulin was purchased from ABCAM and used at working dilution of 1:100. High-resolution images were acquired with a Zeiss LSM 880 confocal microscope equipped with Airyscan superresolution imaging module, using a 63×/1.40 NA Plan-Apochromat Oil DIC M27 objective lens (Zeiss MicroImaging, Jena, Germany)

3.7 Statistics Data were analyzed using analysis of variance (ANOVA) for each region and *post hoc* repeated-measure comparisons (Least Significant Difference (LSD) test). Rejection level was set at $P < 0.05$.

4 RESULTS

4.1 NEK10, RIIβ and PCM1 form a macromolecular complex

A proteomic analysis using full-length RIIβ as bait revealed that the pericentriolar matrix protein 1 (PCM1) and the Nima-related Kinase 10 (NEK10) form a complex with PKA. To verify the interaction between these proteins, I performed a co-immunoprecipitation assay **(Fig. 7a)**. HEK293 cells were transfected for 24 hours with NEK10-flag vector. Lysates were immunoprecipitated with anti-RIIβ or non-immune IgG antibodies and the precipitates were immunoblotted with anti-PCM1, anti-flag and anti-RIIβ antibodies. The data in **figure 7a** show the existence of a trimeric complex composed of PKA, NEK10 and PCM1.I confirmed this interaction using an *in vitro* GST-pull down assays with the recombinant proteins **(Fig. 7b).** I transfected HEK293 cells with NEK10-flag vector and incubated the lysates with purified GST or GST–RIIβ fusion. The precipitates were immunoblotted with anti-PCM1, anti-flag and anti-RIIβ antibodies. **Figure 7b** confirmed the interaction between PCM1, NEK10 and RIIβ proteins.

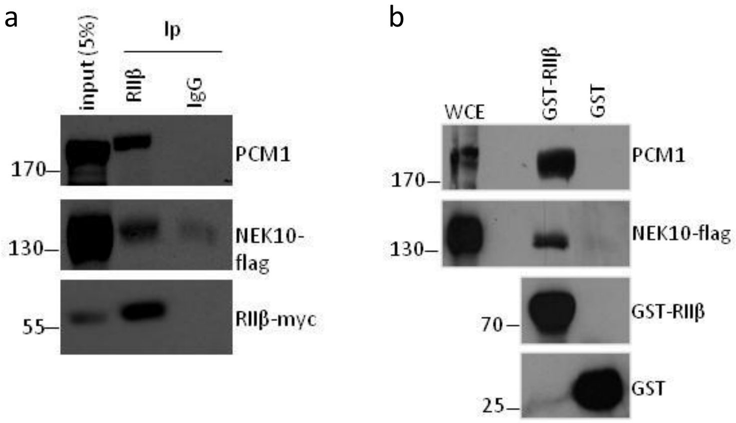


Fig.7 (a) HEK293 cells were transiently transfected with NEK10-flag vector, lysates were immunoprecipitated with anti-RIIβ or non-immune IgG antibodies and immunoblotted with anti-PCM1, anti-RIIβ and anti-flag antibodies. **(b)** HEK293 cells were transiently transfected with NEK10-flag vector; lysates and immunoprecipitates were subjected to pull down assays with purified GST or GST–RIIβ fusion. The precipitates and lysates were immunoblotted with anti-PCM1, anti-flag and anti-GST antibodies.

4.2 Endogenous PCM1, NEK10 and RIIβ colocalize in Human Embryonic Kidney 293 cells

To demonstrate that PCM1, NEK10 and RIIβ are located within the same compartment, I analyzed the localization of the three proteins in HEK293 cells. For this experiment, I performed a triple immunofluorescence assay using anti-PCM1, anti-NEK10 and anti-RIIβ antibodies **(Fig. 8)**. As shown in the figure, immunostaining analysis revealed that the three signals partially colocalize at pericentriolar region, supporting the concept that a fraction of PCM1, NEK10 and RIIβ proteins is restricted within the same intracellular compartment.

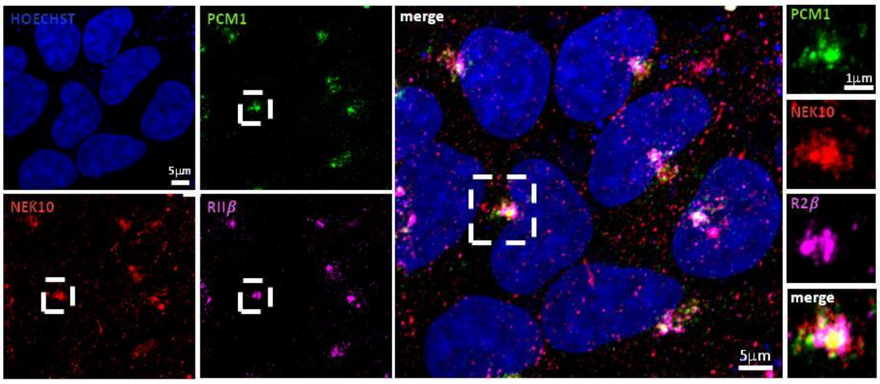


Fig.8 PCM1, NEK10 AND RIIβ colocalize in Human Embryonic Kidney cells. HEK293 were subjected to immunofluorescence assay with goat polyclonal anti-NEK10, rabbit polyclonal anti-PCM1 and mouse monoclonal anti-RIIβ antibodies. The merge composite of the signals shows co-localization of PCM1, NEK10 and RIIβ at pericentriolar region. Scale bar: 5 µm.

4.3 NEK10 is required for ciliogenesis

NEK10 belongs to Nima related kinase family (NEKs) whose members take part to different events underlying to cell cycle progression, centrosome and microtubules formation, and mammalian ciliogenesis. Since these structures are intimately involved both in the assembly of the mitotic spindle and in ciliogenesis[69], we asked if NEK10 also contributes to the primary cilium assembly. Firstly, I demonstrated that NEK10 localizes at primary cilium. For this experiment, I deprived HEK293 cells from serum for 36 hours to induce primary cilium formation. Then, I performed a double immunofluorescence assay using anti-NEK10 and anti-acetylated tubulin antibodies. Acetylated tubulin is a modified variant of tubulin that selectively accumulates along the cilium. The immunostaining analysis shows that a significant amount of NEK10 protein localizes at the base and along the axoneme of primary cilium **(Fig. 9)**

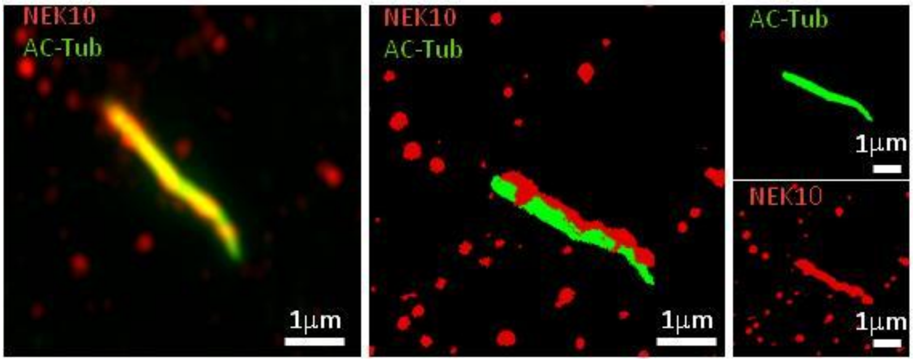
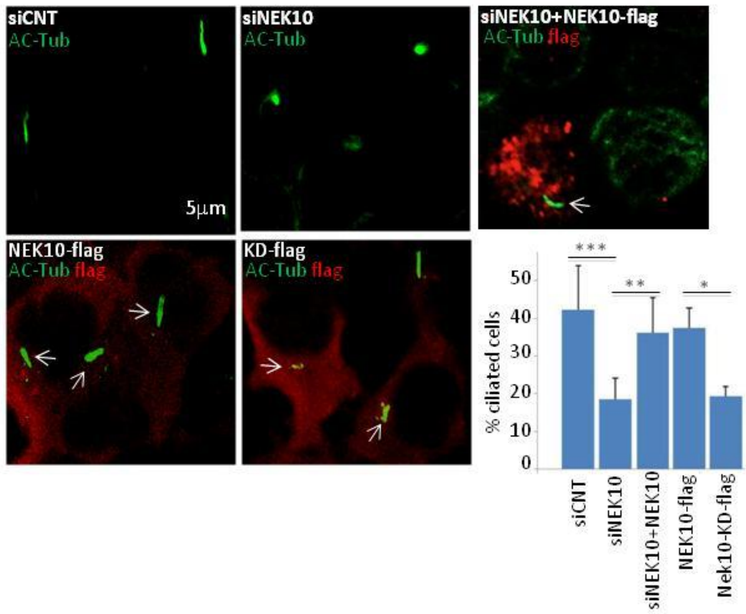


Fig.9 NEK10 localizes at primary cilium. HEK293 cells were serum-deprived for 36 hours and then immunostained for NEK10 (red) and acetylated tubulin (green) antibodies. The signal was analyzed by confocal microscope equipped with Airyscan super resolution imaging module. A merge composite 2D and 3D of the signals shown that NEK10 localized along axoneme of primary cilium.

Once demonstrated the localization of NEK10 in human cells along the primary cilium, I verified the role of NEK10 during primary cilium assembly. To this aim, I transfected transiently HEK293 cells with siRNA targeting endogenous NEK10 and deprived cells from serum for 36 hours. The data shows that, in control (siRNAc) cells, serum deprivation significantly increased the number of primary cilia. In contrast, genetic knock-down of NEK10 drastically reduced the number of ciliated cells. Re-expression of NEK10 reversed the effects of NEK10 silencing, indicating that NEK10 is, indeed, a biologically relevant player of ciliogenesis. **(Fig 10.a)**. Moreover, I repeated the experiment using the kinase-dead mutant of NEK10 (NEK10-KD) carrying an inactivating mutation within the catalytic domain (K548R). As shown in the figure, the NEK10 mutant critically reduced the number of ciliated cells. In the **Fig 10.b** are showed the levels of NEK10 in siRNA transfected cells monitored by immunostaining.

a



b

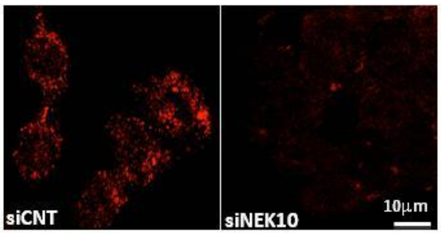


Fig.10 (a) NEK10 is required for ciliogenesis. HEK293 cells were transiently transfected with control (siCNT) or with siRNAs targeting NEK10 (siNEK10), serum deprived for 36 hours, fixed and immunostained with anti-acetylated alpha tubulin and with anti-Flag antibodies (for NEK10-flag). Where indicated, NEK10-flag vector (either wild type or kinase dead, KD) was included in the siRNAs transfection mixture. Arrows indicate the localization of the cilium in cells expressing flag-tagged NEK10. Cumulative data from three independent experiments are shown (lower right panel). **(b)** The levels of NEK10 in siRNA-transfected cells were monitored by immunostaining.

4.4 PKA regulates the stability of primary cilium

The experiments above indicate that NEK10, PCM1 and PKA form a stable complex at pericentriolar region. A fraction of NEK10 localizes at- and regulates primary cilium formation. Previous work demonstrated that a pool of PKA is localized at the base of primary cilium through interaction with an as yet identified scaffold protein[62]. Localization of PKA at cilium is required for cilium formation. However, the impact of PKA activation cilium stability was largely unknown. Accordingly, I investigated the role of PKA activation on cilium stability. HEK293 cells where serum deprived for 36 hours and then stimulated with forskolin (FSK), a diterpene that activates adenylate cyclase (AC), or with isoproterenol (ISO), a beta-adrenergic receptor (bAR) agonist. Cells were subjected to immunostaining analysis using anti-acetylated tubulin antibody. As shown in **Fig.11,** treatment with FSK or Isoprotenerol strongly decreased the number of ciliated cells. These data suggested that activation of cAMP signaling critically impacts on primary cilium stability.

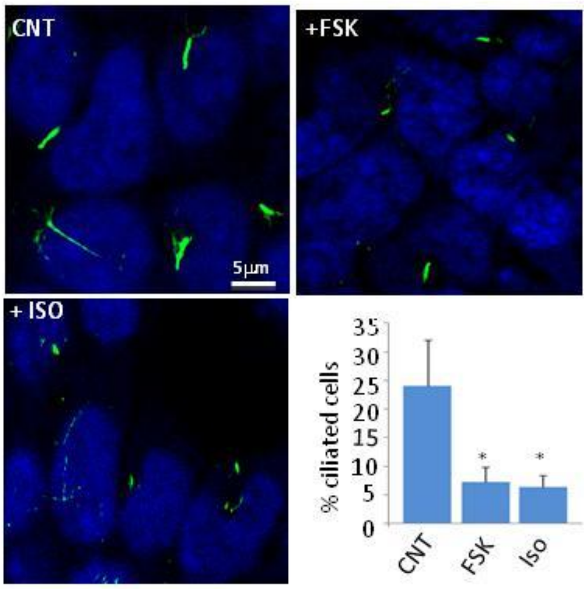


Fig.11 cAMP induced resorption of primary cilium. HEK293 cells were serum deprived for 36h and then left untreated (CNT) or stimulated with isoproterenol (Iso) or forskolin (FSK) for 3 hours. The same cells were immunostained with anti-acetylated tubulin and Draq5. Cumulative data from five independent experiments are shown (lower right panel)

4.5 PKA phosphorylation primes NEK10 for proteolysis via UPS

Our data indicate that NEK10 is required for ciliogenesis, while PKA activation induces cilium resorption. Since NEK10 and PKA are present within the same multimeric complex assembled by PCM1, I tested if/how PKA activation regulates NEK10 levels. To this aim, I monitored the levels of NEK10 in HEK293 cells after the treatment with FSK for 1 hour. The data shown in **Fig.12** revealed that the activation of PKA by FSK caused a severe decrease of NEK10 levels. The effects of forskolin on NEK10 levels were abrogated by pre-treating the cells with the proteasome inhibitor MG132.
The data indicate that, in response to cAMP stimulation, NEK10 undergoes to proteasomal degradation.

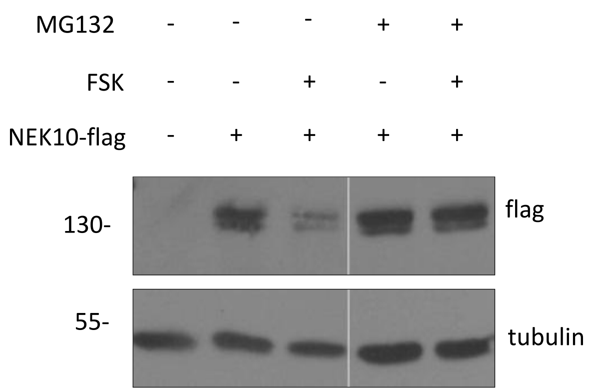


Fig.12 PKA stimulation induces proteolysis of NEK10 by the proteasome. HEK293 cells were transiently transfected with NEK10-flag. After 24 hours, cells were harvested and treated with forskolin (40µM). Where indicated, cells were pretreated with MG132 (20µM/3 hours). Lysates were immunoblotted with anti-flag or anti-tubulin antibodies.

The data above indicate that, in response to cAMP stimulation, NEK10 undergoes proteasomal degradation. We assume that PKA phosphorylation primes NEK10 for proteolysis. Primary sequence analysis of NEK10 predicts two conserved PKA phosphorylation sites (T223 and T812) **(Fig.13).**

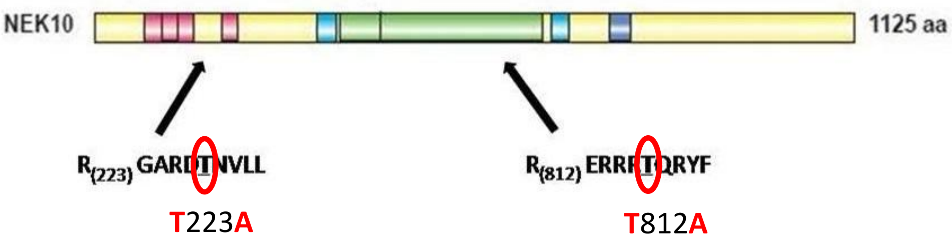


Fig.13 Schematic diagram showing the protein sequence of human NEK10 and the putative PKA consensus sites (thr223 and thr812).

To ask if phosphorylation of one or both of these sites renders NEK10 susceptible to proteolysis, we generated mutant forms of NEK10 using a site-directed mutagenesis to substitute either T223 or T812 with alanine. We tested our hypothesis by analyzing the phosphorylation status of affinity-isolated NEK10 with a PKA substrate antibody. In contrast to phosphorylation of the wild type and T223A NEK10 mutant, the substitution of T812A abolished both basal and FSK-induced NEK10 phosphorylation **(Fig.14)**.

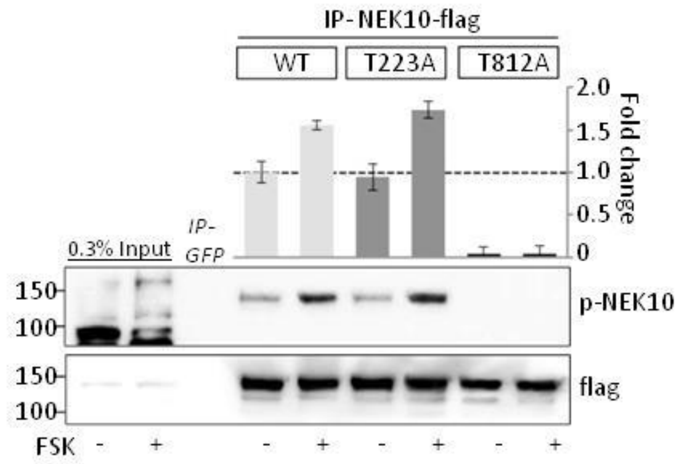


Fig.14 PKA phosphorylates NEK10 at threonine 812. HEK293 cells were transfected with either wild type NEK10-flag or with NEK10-flag mutants (T223A-Flag and T812A-Flag) and were left untreated or stimulated with FSK (40µM715 min). NEK10 was immunopurified with anti-flag antibodies and the precipitates were immunoblotted with anti-flag and anti-phospho-(K/R) (K/R) X(S*/T*) specific antibodies.

Next, we asked if this phosphorylation by PKA is required to induce NEK10 proteolysis. To verify this hypothesis, I evaluated the levels of NEK10 in cells transfected either with NEK10 wild type or with T812A mutant vectors. Both cell lines were stimulated with FSK **(Fig.15a)** or Isoproterenol **(Fig.15b)** for 30 and 60 minutes. The figures show that the phospho mutant (T812A), not sensible to phosphorylation by PKA, is not degradated by FSK and ISO compared to wild type protein.

Ubiquitination is required for proteasomal degradation of a variety of cellular substrates[80]. Accordingly, I asked if cAMP induces ubiquitination of NEK10. To test this hypothesis, I performed ubiquitination assays in HEK293 cells transfected either with hemagglutinin (HA)-tagged ubiquitin and NEK10 (wild type or T812A mutant) and treated with FSK for 60 minutes. The lysates were immunoprecipitates with anti-flag antibody and the precipitates were immunoblotted with anti-HA antibody. **Fig.15c** shows that FSK induces the accumulation of poly-ubiquitinated forms of NEK10, whereas this poly-ubiquitination was abrogated by the T812A mutation. These experiments confirmed that phosphorylation by PKA is necessary to prime proteolysis of NEK10 via UPS.

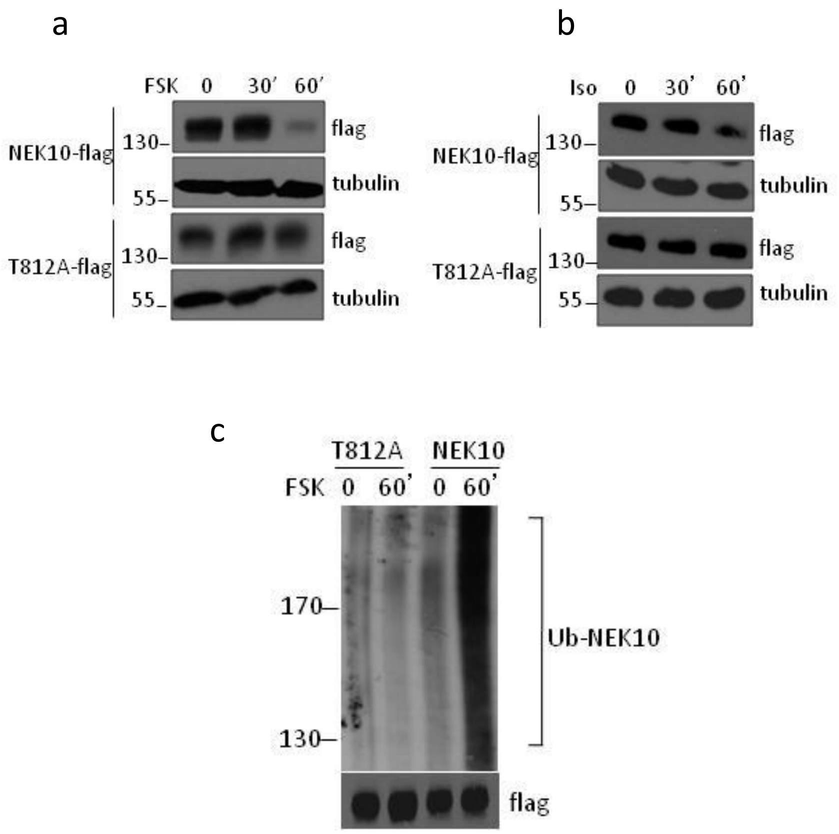


Fig.15 (a) Phosphorylation by PKA drives proteolysis of NEK10. (a-b) Cells transfected with either wild type NEK10-flag or with T812A-flag mutant were left untreated or stimulated with forskolin **(a)** or isoproterenol **(b)** for 30-60 min. Total cell lysates were immunoblotted with anti-flag and anti-tubulin antibodies. **(c)** Cells were transiently co-transfected with NEK10-flag construct (either wild type or T812A mutant) and HA-ubiquitin. Lysates were subjected to immunoprecipitation with anti-flag and immunoblotted with anti-HA and anti-flag antibodies.

Next, I tested if NEK10 phosphorylation was required for primary cilium disassembly induced by the cAMP cascade. Cells were transiently transfected with NEK10 (either wild type or the NEK10-T812A mutant), serum-deprived for two days and then treated with FSK. As shown in **Fig.16,** the T812A mutation prevented FSK-induced cilia disassembly, supporting the concept that PKA phosphorylation of T812 primes NEK10 for proteolysis, which results in cilia disassembly.

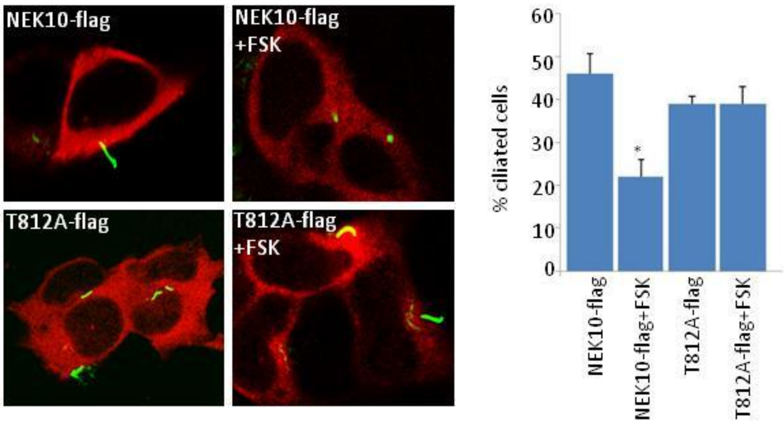


Fig.16. Phosphorylation of NEK10 by PKA induced cilia resorption. HEK293 cells were transfected with either wild type NEK10-flag or with T812A-flag mutant. After transfection, cells were serum deprived for 36h and left untreated or stimulated with FSK (40µM/3 hours). Cells were subjected to a double immunofluorescence for flag (red) and acetylated tubulin (green). Cumulative data from three independent experiments are shown on right panel.

4.6 CHIP is the NEK10 E3 ubiquitin ligase

Since NEK10 is efficiently ubiquitinated under cAMP stimulation, it was necessary to identify the E3 ligase responsible for this ubiquitination. A proteomic analysis identified several PKA partners; one of this is the E3 ubiquitin ligase C-terminus of HSP70 interacting protein (CHIP) known as STUB1. CHIP is a chaperone-associated E3ligase involved in the ubiquitination and degradation of HSP70-bound substrates and contains a tetra-tricopeptide (TPR) motif tandem repeats that mediates interaction with HSP70. First, I verified the interaction between the two proteins. I performed a co-immunoprecipitation assay using lysates from cells transfected with NEK10-flag, HSP70-V5 and CHIP-Myc vectors. The lysates were immunoprecipitated with anti-myc antibody and precipitates were immunoblotted with anti-flag, anti-V5 and anti-myc antibodies. As shown in **Fig.17a** the three proteins form a stable complex in cell lysate.

Then, I tested if the binding between NEK10, CHIP and HSP70 was regulated by cAMP. To this aim, I performed a co-immunoprecipitation assay using lysates from cells transfected either with NEK10 wild type or with NEK-T812A mutant. Cells were induced with FSK for 30 minutes; the lysates were immunoprecipitated with anti-myc antibody and immunoblotted with anti-flag, anti-V5 and anti-myc antibodies. The **Fig.17b-c** shows that the binding between NEK10, HSP70 and CHIP increases after the treatment with FSK, in contrast, the T812A mutation significantly decreases NEK10 binding to CHIP.

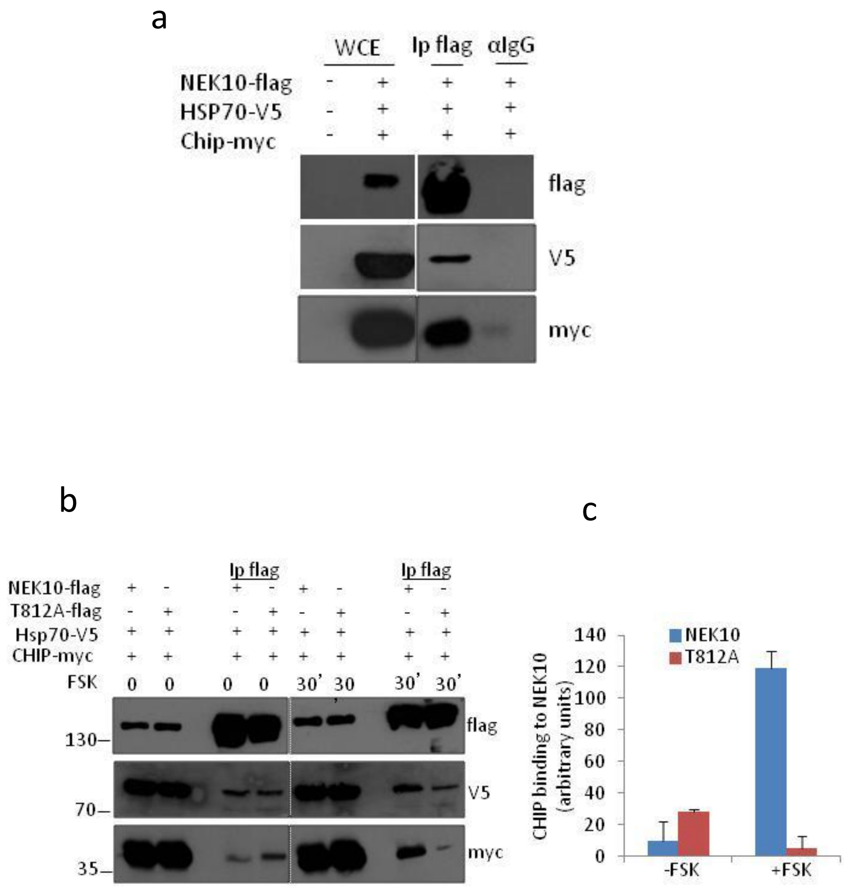


Fig.17 (a) cAMP-induced binding of CHIP to NEK10. HEK293 cells were transiently co-transfected with NEK10-flag, HSP70-V5 and CHIP-myc vectors. To prevent NEK10 degradation by CHIP, cells were treated with MG132 (20µM/8hours) before harvesting. Lysates were immunoprecipitated with anti-flag or with control IgG. The precipitates and lysates were immunoblotted with the indicated antibodies. **(b)** Cells were co-transfected with NEK10-flag vectors (either wild type or T812A mutant), HSP70-V5 and CHIP-myc, serum deprived for 24 hours and then left untreated or stimulated with FSK (40µM/30 min). Lysates were immunoprecipitated with anti-flag antibody and were immunoblotted with the indicated antibodies. **(c)** Cumulative data of three independent experiments shown in b.

We then asked if CHIP degrades NEK10 in the absence of MG132. In the **Fig. 17d** I co-transfected cells with NEK10-flag and CHIP-myc vectors, alternately with wild type or with catalytically inactive mutant (K30A) of CHIP that does not bind HSP70. The lysates were immunoblotted with anti flag, myc and tubulin antibodies. The figure shows that in presence of CHIP wild type there is a decrease of NEK10 levels whereas CHIP-K30A mutant in not able to degradate NEK10.

In the **Fig17.e** I performed an ubiquitination assay using cells transfected with control siRNAs or siRNAs targeting endogenous CHIP. After transfection, cells were left untreated or stimulated with Isoprotenerol for 1 hour. The figure shows that in basal condition there is an accumulation of poly-ubiquitinated form of NEK10 ISO dependent, by the contrast, the genetic knock-down of endogenous CHIP prevented ISO-induced NEK10 polyubiquitination. These findings supported the idea that PKA controls NEK10 stability through CHIP.

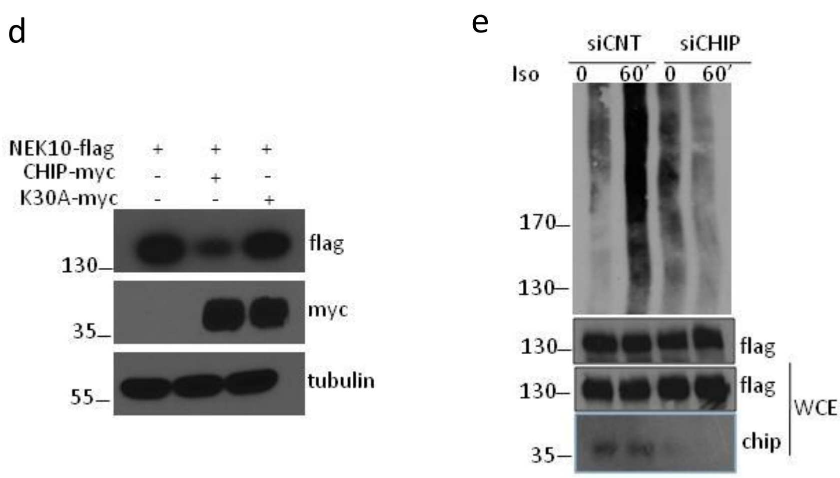


Fig.17 CHIP ubiquitylates NEK10. (d) Lysates from cells co-transfected with NEK10-flag and CHIP (either wild type or K30A mutant) were immunoblotted with the indicated antibodies **(e)** Cells co-transfected with HA-ubiquitin, NEK10-flag and siRNAs (either control siRNA or siCHIP) were serum-deprived overnight and stimulated with isoproterenol for 1 hour. Lysates were subjected to immunoprecipitation with anti-flag antibody. Ubiquitinated NEK10 was revealed by immunoblot with anti-HA antibodies.

Once determined that cAMP primes proteolysis of NEK10 through the interaction with CHIP, we verified if CHIP mediates the effects of cAMP on cilia stability. To this aim, I transfected HEK293 cells with control siRNAs or siRNAs targeting endogenous CHIP. Twenty hours after the transfection I deprived the cells from serum for 36 hours and treated them with FSK for 3 hours. Cells were subjected to immunofluorescence assay with anti-acetylated tubulin antibody. As shown in the figure **(Fig 17f)**, down regulation of endogenous CHIP prevented cilia resorption induced by FSK treatment.

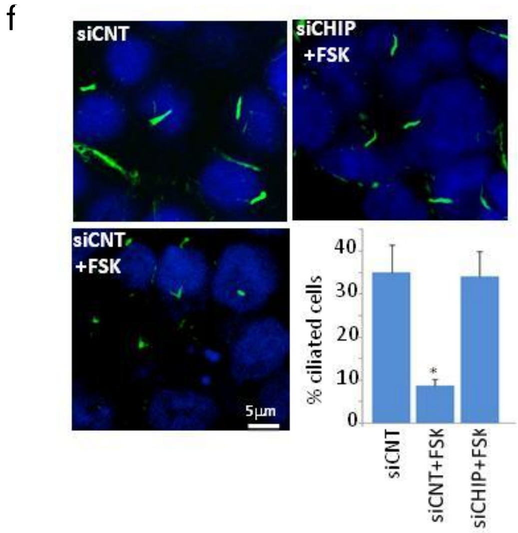


Fig.17 CHIP ubiquitylates NEK10 and mediates cAMP effects on primary cilium stability (f) Cells were transiently transfected with control or with siRNAs targeting CHIP, serum-deprived for 36h and then left untreated or stimulated with FSK (40μM) for 3 hours. Primary cilia were visualized by immunostaining with acetylated tubulin antibody whereas nuclei with Draq5. Cumulative data from five independent experiments are shown in the graph near the figure.

4.7 Dysregulation of CHIP affects cilia in SCAR16 disease

Biallelic *STUB1* mutations resulting in aberrant CHIP have been identified in patients with clinical features of autosomal recessive spinocerebellar ataxia-16 (SCAR16). This is a rare genetic syndrome characterized by truncal and limb ataxia resulting in gait instability, mild peripheral sensory neuropathy, and cognitive defects. Hypogonadism can also be present in these patients (Gordon Holmes syndrome, GHS), consistent with signaling defects and altered responses to hypothalamic hormones. Mice lacking *STUB1*/CHIP gene show a phenotype that recapitulates most of the SCAR16 features[81]. Accordingly, we determined if CHIP mutations affect primary cilia. We analyzed ciliogenesis in primary fibroblasts isolated from cutaneous biopsies of SCAR16 patients or from healthy volunteers. **Fig.18** shows that FSK treatment in normal fibroblasts promoted resorption of cilia. In contrast, no major effects of FSK stimulation on cilia were evident in SCAR16 fibroblasts. Interestingly, genetic silencing of NEK10 in SCAR16 fibroblasts markedly reduced the number of ciliated cells, even in the absence of FSK, further supporting a role of the CHIP-NEK10 axis in control of cilium stability.

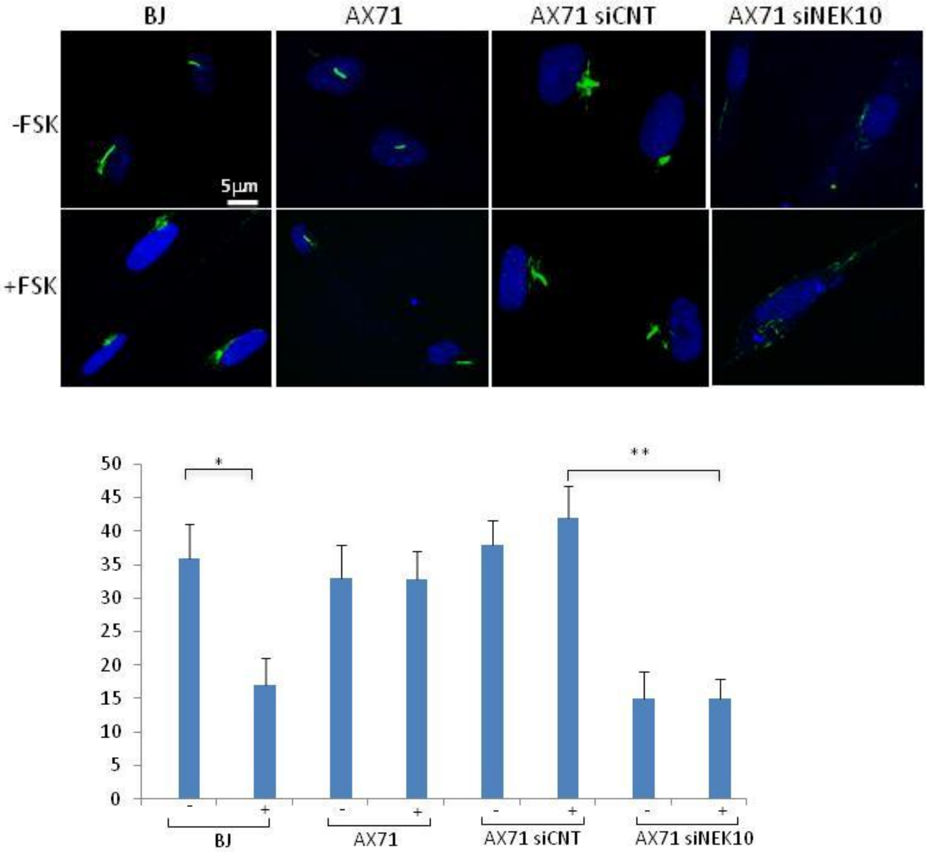


Fig.18 CHIP, NEK10 and cilia in SCAR16 fibroblasts. Skin fibroblasts from healthy volunteers (BJ) and SCAR16 patients (AX71) were serum deprived for 48h and treated with FSK (80 μM/6h). Cells were fixed and stained for acetylated tubulin and Draq5. Where indicated, AX71 cells were transiently transfected with control siRNA or with siRNA targeting endogenous NEK10, before stimulation. Cumulative data from 4 independent experiments are shown.

5 DISCUSSION AND CONCLUSION

Primary cilia are considered very important organelles that emanate from cell surface and are able to detect external signals and reintroduce them into cells. Primary cilia are present on the apical surface of the majority of cells in the human body and the structure that promotes the assembly of primary cilium is the centrosome, the principal microtubule organizing center (MTOC) in animal cells. The cilium is composed by axoneme and the basal body[53].

The primary cilium is a compartmentalized complex for signal integration and propagation relevant for many developmental processes. In dividing cells, the transition between centrosome and primary cilium is functionally linked. In mitotic interphase, centrosomes organize the cytoplasmic microtubule network, whereas in mitosis they regulate mitotic spindle dynamics and cytokinesis. In postmitotic cells, the centrosome migrates to the cell surface, and one of the centrioles differentiates into a basal body from which microtubules nucleate to form a primary cilium. In normal proliferating cells, the cilium can be transiently observed in G1 phase, disappearing when the cell enters the cell cycle[82]. A significant fraction of PKA is localized at the base of cilium through interaction with AKAPs, controlling essential aspects of ciliogenesis and the Hedgehog (Hh) pathway. However, the impact of PKA activation on the turnover of ciliary proteins and its role in primary cilium stability were largely unknown.

As mentioned above, there is a thigh connection between cAMP cascade and UPS system and derangements in these mechanisms are linked to arise several neurodegenerative and proliferative disorders.

In the thesis, I reported the identification of the serine/threonine kinase NEK10 as a novel positive regulator of ciliogenesis. NEK10 is member of the Nima-related kinases activated at

G2/M transition and its activity is required for proper cell cycle progression. I demonstrated the existence of a macromolecular complex in which NEK10 is localized at the primary cilium through the interaction with PKA and PCM1, a pericentriolar scaffold protein involved in different aspects of microtubule dynamics, cell division and ciliogenesis. I found that NEK10 protein stability is a critical determinant for the assembly/disassembly of cilium and it is mediated by the GPCR signaling. By a combinatorial approach of biochemistry, cell biology and molecular genetics, I demonstrate the presence of a trimeric complex composed by NEK10, PCM1 and regulatory subunit of PKA (RIIβ) at pericentriolar region of mammalian cells. In particular, I found that NEK10 localizes at the base and along the axoneme of primary cilium. NEK10 down regulation severely affected the assembly of primary cilium.

I also analyzed the intersection between GPCR signaling and primary cilium. I found that NEK10 is a novel direct target of PKA. Phosphorylation of NEK10 by PKA at Thr812 primes NEK10 to ubiquitination and proteolysis. Disappearance of NEK10 promotes cilia resorption. A proteomic analysis allowed the identification of CHIP as the E3 ubiquitin ligase that binds to- and ubiquitylates NEK10, causing NEK10 proteolysis through the UPS and cilia resorption. Removal of CHIP prevented cAMP effects on cilium resorption. These findings point to CHIP as a novel regulator of protein turnover at ciliary sites that efficiently couples GPCR signaling to cilia dynamics. This mode of regulation was further supported by evidence that germline inactivating mutations of CHIP that cause SCAR16 disease prevented cAMP-induced disassembly of cilia.

Altogether, the findings reported in my thesis elucidate the mechanism(s) underlying cilia resorption during GPCR stimulation, both in healthy and disease conditions. They also provide mechanistic insights into how cAMP controls cell growth. It is well established that the cAMP cascade regulates growth and differentiation of a wide variety of cell types. PKA activation can either induce or inhibit cell growth, depending on cell type or metabolic conditions[83]. In growth-arrested endocrine cells, the cAMP-PKA pathway promotes the transition from G0 to G1 phase, allowing the cells to progress through the cell cycle[84]. The transition from quiescent to proliferative state requires disassembly of the primary cilium.

Several targets of PKA have been identified and causally linked to induction of cell growth. However, if and how PKA activation modulates the activity of proteins controlling cilia stability in starved cells was largely unexplored. These findings help to define the relevance of PKA pathway in cilia resorption in the course of hormone stimulation. We show that PKA activation by cAMP agonists targets NEK10 for proteolysis through the UPS. The cAMP cascade induces cilia disassembly and promotes entry into the cell cycle by removing the NEK10 pro-ciliogenic kinase. NEK10 thus represents a nodal point in the ciliary compartment where cAMP signaling and the UPS converge and integrate to control essential aspects of cilia dynamics and, most likely, cell growth. Mutations affecting any component of this proteolyitc machinery may alter the sensitivity of the cells to hormones or growth factors, profoundly impacting on cell growth and vertebrate development.

Although the results of my thesis enhance the role of cAMP into disassembly of primary cilium, there are some points that need to be addressed. It is important to understand whether cilia resorption induced by cAMP has a physiologically relevant implications for cell biology. Previous work revealed that activation of cAMP pathway promotes ciliogenesis.[85, 86].

This apparent discrepancy could not be ascribed to the different cell models used, since we confirmed that in serum supplemented, confluent cells cAMP stimulation had no major impact on cilum stability (data not shown). These findings suggest that cAMP pathway may have a dual effect on primary cilia depending on how growth arrest is achieved. In the presence of serum, cAMP contributes to primary ciliogenesis induced by cell confluency, while under serum starvation the same messenger promotes cilium disassembly.

Finally, the findings reported in my thesis indicate that NEK10 is a new ciliary protein that in concert with other centriolar proteins plays a major role in the regulation of assembly/disassembly of primary cilium.

My next goal is the identification and the molecular characterization of the relevant NEK10 substrates involved in mammalian ciliogenesis. Derangement of this NEK10-regulated signaling circuitry may underpin to genetic and proliferative disorders human disorders.

In conclusion, I have identified a PCM1-centered multimeric complex that functionally links second messenger signaling (cAMP), kinase activities (PKA, NEK10) and the UPS (CHIP) to cilia dynamics. This mechanism explains how compartmentalized signaling networks regulate cilia formation in both physiological and pathological conditions.

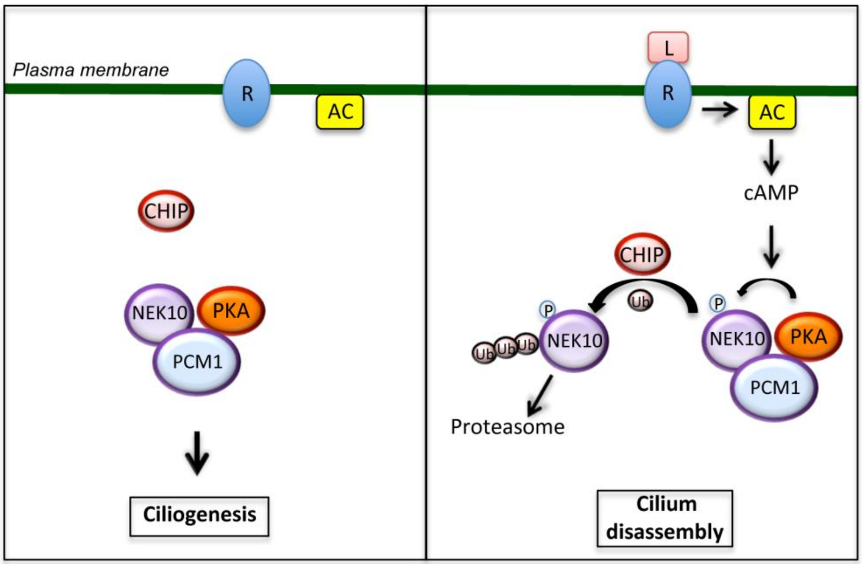


Figure 19. Molecular mechanism model. Under resting conditions, PKA holoenzyme form a stable complex with PCM1 and NEK10, promoting ciliogenesis. Elevation of intracellular cAMP levels by ligand (L) stimulation of the adenylate cyclase (AC) efficiently activates PKA which phosphorylates NEK10. Phosphorylation primes ubiquitin-dependent proteolysis of NEK10 by E3 ligase CHIP. The decrease of NEK10 levels promotes the resorption of primary cilium.

6 APPENDICES

AC Adenylyl cyclase

GPCR G protein coupled receptor

PKA Protein kinase A

PDE Phosphodiesterase

UPS Ubiquitin Proteasome System

CHIP C-terminus of HSC70 interacting protein

FSK Forskolin

ISO Isoprotenerol

cAMP cyclic AMP

EPAC RAP exchange proteins

cNGC cAMP gated ion channels

AKAP A-Kinase-Anchor-Proteins

TPR tetratricopeptide repeats domain

SCAR16 spinocerebellar ataxia autosomal recessive 16

PCM1 pericentriolar matrix protein 1

NEK10 Nima-related Kinase 10

RIIβ 51egulatory subunit of PKA

Hh Hedgehog

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9 LIST OF PUBLICATIONS

- Compartmentalized Camp Signaling In Neurodegenerative Diseases
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